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US ARMY MEDICAL RESEARCH LABORATORY

FORT KNOX, KENTUCKY

REPORT NO. 622

EFFECT OF ENZYMATIC FUNCTIONAL GROUP INHIBITORS ON PROTEOLYTIC ENZYMES OF CROTALUS ATROX VENOM

by

John H. Brown, Ph. D.

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In conducting the research described in this report, the investigators adhered to the "Principles of Laboratory Animal Care as established by the National Society for Medical Research."

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ABSTRACT

EFFECT OF ENZYMATIC FUNCTIONAL GROUP INHIBITORS ON PROTEOLYTIC ENZYMES OF CROTALUS ATROX VENOM

OBJECT

To determine the effects of several functional group-specific inhibitors of enzymes and of metal ions on the activity of the proteolytic enzymes of Crotalus atrox venom. To attempt to discern the type and number of proteolytic enzymes present in crude venom and to obtain a detoxified, yet immunologically active venom.

RESULTS

Only caseinolytic activity seems to be appreciably inhibited by sulfhydryl group-specific reagents. All proteases are inhibited by thiol-containing compounds. Diisopropylfluorophosphate inhibits only proteolytic activity with N-benzoyl-DL-arginine-p-nitroanilide as substrate. N-bromosuccinimide inhibits only proteolytic activity on congocoll and N-benzoyl-DL-arginine-p-nitroanilide. Ethylenediaminetetraacetate inhibits proteolytic activity on all substrates tested except N-benzoyl-DL-arginine-p-nitroanilide. ε-Amino-n-caproic acid enhances caseinolysis at low concentrations and inhibits at higher concentrations. Calcium ions and other divalent alkaline earths have a general activating effect on proteolytic activity of venom. Heavy metal ions, in general, have an inhibitory effect on proteolytic activity.

CONCLUSIONS

The utilization of some of the functional group-specific reagents in possible production of detoxified, immunochemically responsive venom should be determined. Some of these reagents should be studied for their usefulness in local treatment of snakebite.

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EFFECT OF ENZYMATIC FUNCTIONAL GROUP INHIBITORS ON PROTEOLYTIC ENZYMES OF CROTALUS ATROX VENOM

INTRODUCTION

It is generally known that most Crotalid snake venoms manifest proteolytic activity. Several investigators have reported the presence of more than one proteolytic enzyme in some snake venoms (1-8).

The importance of proteolytic enzymes of certain snake venoms is found in their relationship to the toxicity of venom. Maeno et al. (9-12) have demonstrated that proteolytic enzymes and phospholipase A are responsible for myolysis produced by an intramuscular injection of Habu venom into animals. One of the proteolytic enzymes in this venom has been shown to produce myolysis with hemorrhage, whereas phospholipase A has been found to cause significant myolysis and edema without hemorrhage (12). Recently, Ohsaka et al. (5) provided evidence for the presence of at least two hemorrhagic principles of Habu venom, both of which were associated with proteolytic activity. Several other investigators have sought correlations between the toxicity of snake venoms and their enzymic activities (3, 4, 13-21).

Biochemical knowledge of the nature of enzymes of snake venoms is exiguous. To date, few investigators have concerned themselves with elucidation of types of proteolytic enzymes in snake venoms, functional groups responsible for their enzymic activity or possible metal ion activators or inhibitors.

If proteolytic enzymes or other enzymes present in snake venom are responsible for toxicity of certain snake venoms, and these enzymes can be inhibited by chemical reagents, it is possible that such chemical reagents (or their non-toxic analogs, if toxic themselves) might be used as drugs in clinical treatment of snake envenomation, either alone or in combination with established methods of treatment (e. g., incision and suction, antivenin). One such enzymic inhibitor (ethylenediaminetetraacetate) is currently being used as an antitoxin in treatment of Habu envenomation (22). It might also be possible to inactivate certain toxic principles of snake venom by chemical means and yet maintain normal immunologic properties, as in the case of certain enzymes (23, 24).

This report is concerned with effects of several well-known functional group inhibitors of enzymes and with effects of metal ions on the

activity of proteolytic enzymes of Crotalus atrox venom. By using more than one substrate to assay proteolysis in the presence of different enzymic inhibitors one can demonstrate how different proteolytic enzymes in crude venom might be distinguished without prior separation. Combined use of enzymic inhibitors and several different substrates should also yield information concerning the specificity and mode of action of these proteases. This report also lists data on two proteolytic enzyme substrates which have not previously been used to assay snake venom proteases.

MATERIALS AND METHODS

Pooled Crotalus atrox venom was collected over ice from several hundred rattlesnakes, immediately frozen and lyophilized within two weeks after collection and stored in the cold in a desiccator. Immediately before use the venom was reconstituted by dissolving it in distilled water or an appropriate buffer and centrifuged to remove undissolved matter.

Casein was prepared using the method of Dunn (25).

All of the chemicals used in these studies were reagent grade.

Analytical Methods. Protein content of venom was measured using the biuret method of Gornall et al. (26).

Proteolytic activity on casein was determined by means of the Kunitz test (27). The 1.0% casein solutions were prepared in 0.05 M Tris buffer, pH 8.0. After the undigested protein was precipitated with 3.0 ml of 5.0% trichloracetic acid, it was allowed to stand at 37° for 30 minutes, then filtered through Whatman #50 filter paper. The proteolytic activity was measured in terms of absorbancy (Beckman DU Spectrophotometer) at 280 mμ with blanks from which 1.0 ml of venom was omitted, Figure 1.

Proteolytic activity on congocoll was determined using a modified procedure of Nelson, Ciaccio, and Hess (28). One-half ml of venom solution in 0.05 M Tris buffer, pH 8.0, was added to 10.0 mg of congocoll in a test tube. After incubation for 30 minutes at 37°, contents of the tubes were diluted to 10.0 ml by addition of 9.5 ml of buffer, then filtered through Whatman #50 filter paper. Absorbancy of the colored solution was determined at 495 mμ using a Beckman Model B Spectrophotometer, Figure 2.

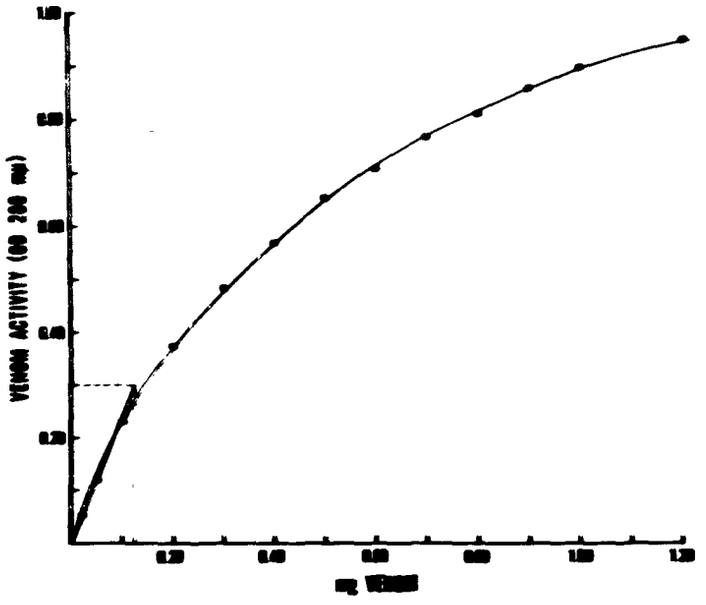

Fig. 1. Standard curve for the hydrolysis of 1.0% casein by proteases of Crotalus atrox venom at pH 8.0 in 0.05 M Tris buffer. Absorbancy at 280 mμ plotted vs. milligrams of venom.

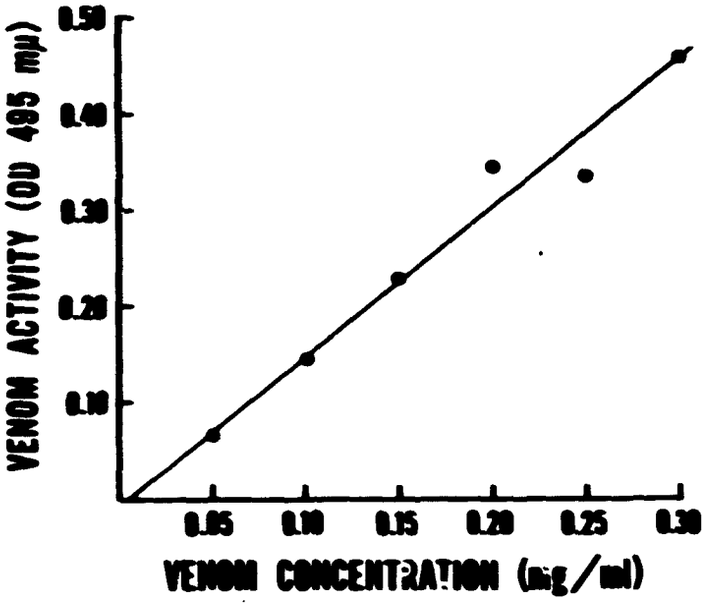

Fig. 2. Standard curve for the release of soluble colored products from 10.0 mg of dye-cell at pH 8.0 in 0.05 M Tris buffer by the action of proteases of Crotalus atrox venom. Absorbancy at 495 mμ plotted vs. concentration of venom (mg/ml).

Proteolytic activity on azocoll was determined by the method of Ridwell and Van Heyningen (29) as modified by Ohsaka (6) using 0.2 ionic strength sodium phosphate buffer, pH 7.4, prepared by combining and diluting to 2.0 liters, 0.5, 24.3, 72.0 ml of 4.0 M sodium dihydrogen phosphate, 0.5 M disodium hydrogen phosphate and 5.0 M sodium chloride, respectively. Two-tenths ml of venom solution was added to 20.0 mg azocoll and 5.0 ml of phosphate buffer. The mixture was incubated for one hour at 37° with occasional shaking. The reaction was stopped by filtering off the remaining substrate with Whatman #50 filter paper. The intensity of color of the resulting filtrate was measured at 520 mμ with a Beckman Model B Spectrophotometer. The relationship between venom concentration and enzymatic activity is shown in Figure 3.

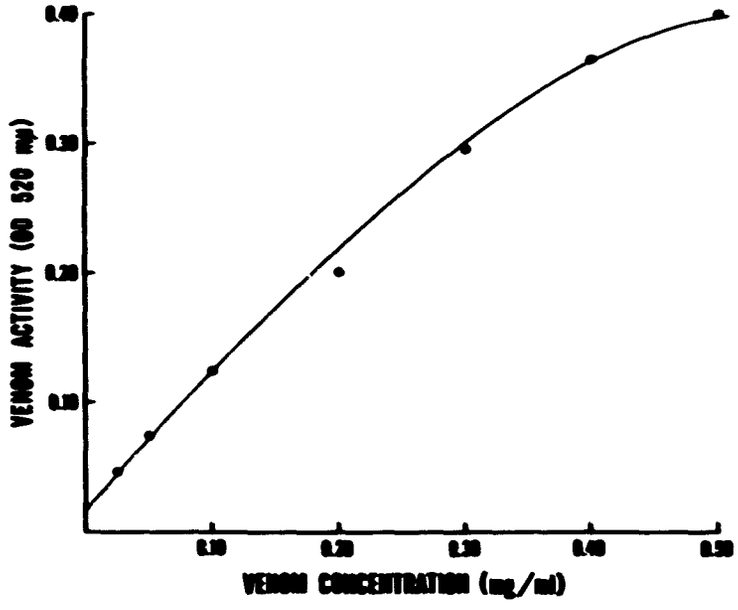

Fig. 3. Standard curve for the release of soluble colored products from 20.0 mg of azocoll at pH 7.40 in sodium phosphate buffer by the action of proteases of Crotalus atrox venom. Absorbancy at 520 mμ plotted vs. concentration of venom (mg/ml).

Proteolytic activity on N-benzoyl-DL-arginine-p-nitroanilide (BAPNA) was determined by a modified method of Haverback et al. (30). One ml of venom solution was added to 1.5 ml of BAPNA (1.0%) and 0.5 ml of 0.1 M Tris buffer, pH 8.0, which had been incubated for 5 minutes at 37°. After 30 minutes, 1.0 ml of 0.2 N HCl was

added to stop the reaction. Increase in optical density, Figure 4, at 383 mμ was used to indicate proteolytic activity.

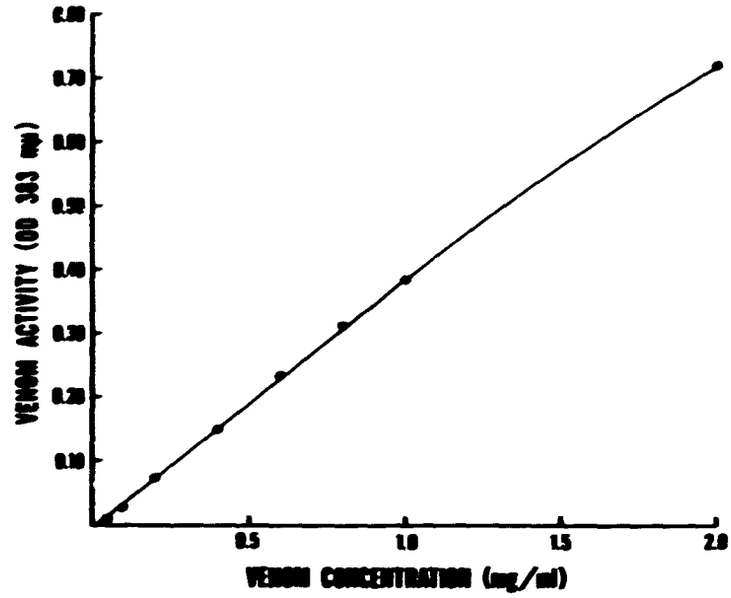

Fig. 4. Standard curve for the hydrolysis of 15.0 mg of BAPNA at pH 8.0 in 0.10 M Tris buffer. Absorbancy at 383 mμ plotted vs. concentration of venom (mg/ml).

Proteolytic activity on fibrin blue was measured in a manner similar to that used for congocoll, using 0.2 ionic strength sodium acetate buffer. No activity could be detected using the same concentrations of venom as were used in the congocoll test.

RESULTS

pH Optima. Figures 5-7 illustrate proteolytic activities of venom as a function of pH in different buffers. Proteolytic activity measured on azocoll, Figure 5, shows a pH optimum at 8.0 in 0.10 M Tris buffer. With BAPNA as substrate for proteolytic activity the pH optimum, Figure 6, has a broad range with maximal activity at 8.5 in sodium veronal buffer ($\mu = 0.2$). The activity at pH 9.0 in sodium veronal is about the same as that in glycine buffer ($\mu = 0.2$), pH 9.5.

The optimal pH for proteolysis measured in 0.05 M Tris buffer with congocoll as substrate is 9.0, Figure 7 (page 7). The almost two-fold

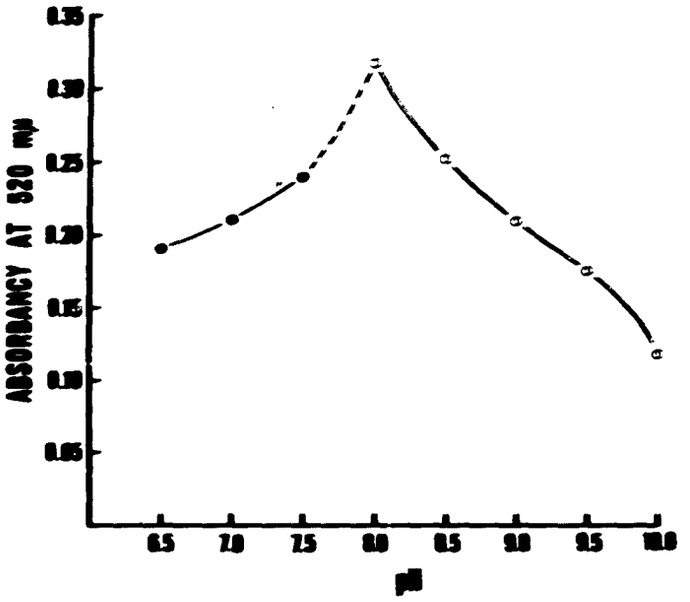

Fig. 5. Effect of pH on the release of soluble colored products from 20.0 mg of azocoll by the action of proteases of Crotalus atrox venom. Closed circles, 0.10 M sodium phosphate buffer; open circles, 0.10 M Tris buffer. Absorbancy at 520 mμ plotted vs. pH.

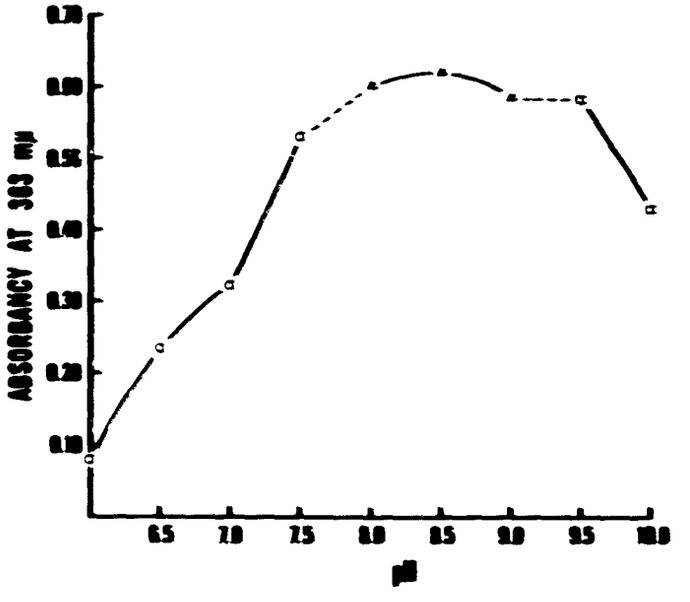

Fig. 6. Effect of pH on the hydrolysis of BAPNA by proteases of Crotalus atrox venom. Open circles, 0.02 M sodium phosphate buffer; open triangles, 0.02 M sodium veronal buffer; open squares, 0.02 M glycine buffer. Absorbancy at 383 mμ plotted vs. pH.

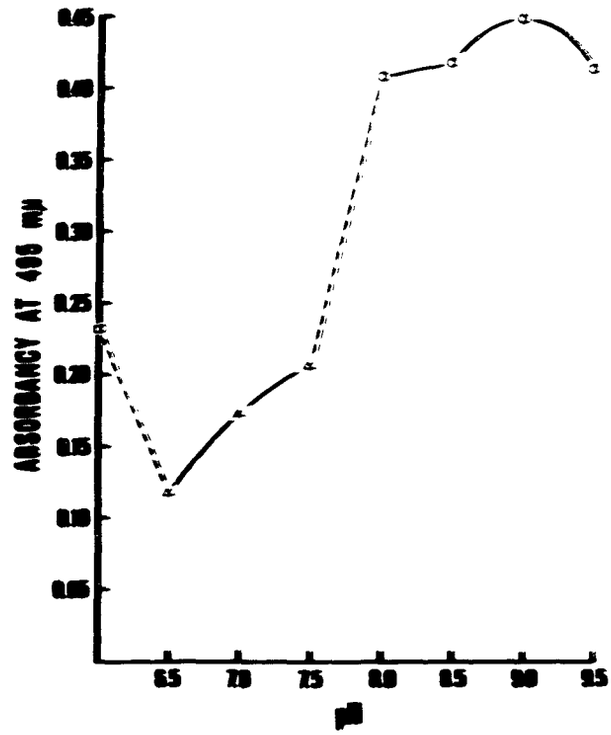

Fig. 7. Effect of pH on the release of soluble colored products from congocoll by the action of proteases of Crotalus atrox venom. Open square, 0.05 M sodium acetate buffer; open triangles, 0.05 M sodium phosphate buffer; open circles, 0.05 M Tris buffer. Absorbancy at 495 mμ plotted vs. pH.

difference in proteolytic activity between pH 7.5 in 0.05 M sodium phosphate buffer and pH 8.0 in 0.05 M Tris buffer might indicate that phosphate inhibits proteolytic activity on congocoll.

Effect of Dialysis. As shown in Table 1, about 10% of the biuret reacting material in venom is removed during 16-18 hours dialysis in the cold with 500 ml of 0.05 M Tris buffer, pH 8.0. Even though about a 10% loss in protein occurs during dialysis, proteolytic activity on casein increases 31%. Dialysis with 0.05 M Tris buffer, pH 8.0, containing 1.0 mM ethylenediaminetetraacetate (EDTA) results in about an 18% loss in protein and a 63% loss in proteolytic activity on casein. Addition of up to a two-fold excess of calcium or other metal ions, with respect to EDTA, to venom that had been dialyzed overnight in the cold with 0.05 M Tris buffer, pH 8.0, containing 1.0 mM EDTA was ineffective in reversing the inhibition caused by EDTA.

TABLE I

Effect of Dialysis on Protein Content and Proteolytic Activity of Crotalus atrox Venom

Venom (10.0 mg/ml) was dialyzed overnight at 2° with 500 ml of 0.05 M Tris buffer, pH 8.0, or Tris containing 1.0 mM EDTA. The values (including the average deviation) represent 10-12 experiments. Proteolysis was measured using the Kunitz test (27).

	Protein		Volume		Activity	
Dialysis Medium	Before %	After %	Before %	After %	Before %	After %
Tris	100.0	90.4±3.8	100.0	102.3±1.6	100.0	131.0±6.0
Tris + EDTA	100.0	81.8±7.1	100.0	99.0±1.3	100.0	37.0±6.0

Effect of Metal Ions. The data in Figure 8 show the stabilizing effect of calcium ions on autodigestion of proteolytic enzymes of dialyzed venom; there is also activation of the proteolytic enzymes by calcium ions. Although a considerable amount of autodigestion of caseinolytic enzyme(s) occurs within 60 minutes, addition of calcium ions to a final concentration of 22 mM restores activity to its original level. Dialyzed venom loses about 50% of its caseinolytic activity in 2 to 3 hours under similar conditions. Calcium ions also appear to be potent activators of the caseinolytic enzymes in this venom, Figure 9. Table 2 (page 10) and Figure 10 (page 11) summarize some of the experiments of effects of metal ions on proteolytic enzymes using different substrates. The alkaline earths seem to have a general activating effect on the proteolytic enzymes with the following exceptions: magnesium ions inhibit using BAPNA as substrate; barium ions do not activate caseinolysis. Manganese ions inhibit proteolytic activity except when using azocoll as substrate. Divalent cobalt, iron, nickel, lead, mercury, cadmium, copper, and zinc ions inhibit proteolytic activity to varying degrees with the following exceptions: zinc, iron, and nickel ions do not inhibit proteolysis using BAPNA as a substrate; lead ions do not inhibit caseinolysis.

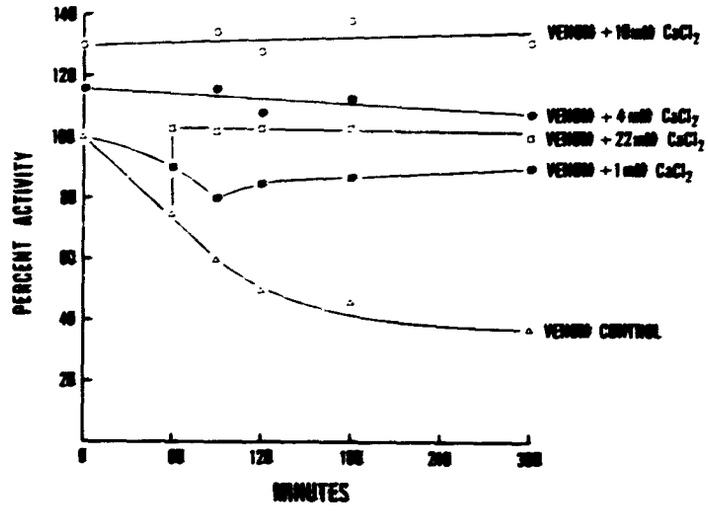

Fig. 8. Stabilization of caseinolysis by proteases of Crotalus atrox venom. Dialyzed venom (1.0 mg/ml) was incubated at 37° in 0.05 M Tris buffer, pH 8.0, in the presence of various concentrations of calcium chloride. One-half ml aliquots were assayed for caseinolysis at various intervals of time using the Kunitz test (27). Venom controls were incubated in the absence of calcium chloride.

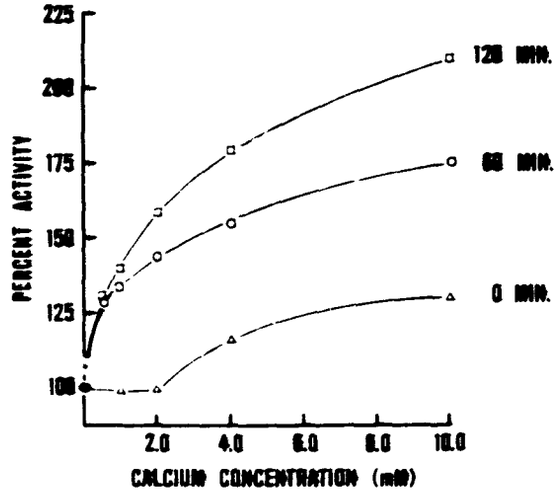

Fig. 9. Effect of calcium chloride on the caseinolytic activity of Crotalus atrox venom. Dialyzed venom (1.0 mg/ml) was incubated at 37° in 0.05 M Tris buffer, pH 8.0, with several different concentrations of calcium chloride. One-half ml aliquots were removed and assayed for caseinolytic activity using the Kunitz test (27).

TABLE 2

Effect of Metal Ions on Proteolytic Activity of
Crotalus atrox Venom

Venom and the metals listed were incubated at 37° for 0 and 60 minutes in appropriate buffers then assayed. The final concentrations of venom in the incubation mixtures for assay with azocoll, BAPNA, congocoll, and casein were 0.5, 1.0, 0.25, and 0.5 mg per ml, respectively (see Materials and Methods).

		BAPNA Per Cent Activity		Congocoll Per Cent Activity		Azocoll Per Cent Activity		Casein Per Cent Activity	
Metal	mM	0 Min	60	0 Min	60	0 Min	60	0 Min	60
None	-	100	98	100	83	100	83	100	75
$MgSO_4$	5	45	69	108	110	115	113	118	111
"	10	50	69	-	-	147	124	139	128
$CaCl_2$	5	98	116	131	112	126	117	125	114
"	10	100	124	-	-	127	138	141	129
$BaCl_2$	5	101	121	109	107	140	118	96	73
"	10	-	-	-	-	138	127	96	73
$MnCl_2$	5	51	70	84	57	97	101	89	50
"	10	49	64	-	-	-	84	44	12
$CoCl_2$	5	38	57	50	40	63	47	52	68
"	10	38	57	-	-	53	40	-	-
$FeSO_4$	5	138	138	96	60	84	64	-	-
"	10	141	146	-	-	77	43	-	-
$NiCl_2$	5	126	144	81	10	93	27	-	20
"	10	-	-	-	-	66	15	53	10
$Pb(oAc)_2$	5	87	87	82	55	95	75	111	91
"	10	77	75	-	-	71	62	69	75
$HgCl_2$	5	5	26	43	19	-	-	19	7
"	10	0	29	-	-	13	25	6	2
$CdCl_2$	5	72	83	20	7	54	38	4	1
"	10	63	78	-	-	55	26	1	1
$CuCl_2$	5	-	-	34	8	43	35	35	9
"	10	80	78	-	-	42	33	4	4
$ZnSO_4$	5	88	112	67	59	87	80	32	25
"	10	-	-	-	-	71	65	8	8

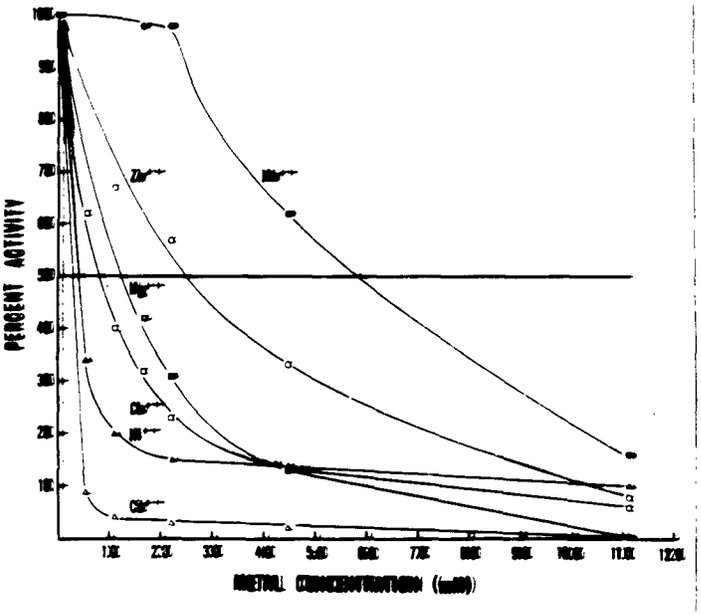

Fig. 10. Effect of heavy metal ions on the caseinolytic activity of Crotalus atrox venom. Dialyzed venom (1.0 mg/ml) was incubated at 37° in 0.05 M Tris buffer, pH 8.0, containing various concentrations of Mn^{++}, Zn^{++}, Hg^{++}, Cu^{++}, Ni^{++}, and Cd^{++} for 60 minutes. One-half ml aliquots were then removed and assayed for caseinolytic activity using the Kunitz test (27).

Effect of Enzyme Inhibitors. The data in Table 3 indicate the effects of various inhibitors on proteolytic activity. Only caseinolytic activity seems to be appreciably inhibited by the sulfhydryl group reagents tested. All proteases seem to be inhibited by thiol group-containing compounds; British anti-lewisite (BAL), however, does not inhibit proteolytic activity with BAPNA as substrate. Diisopropylfluorophosphate (DFP) inhibits only proteolytic activity on BAPNA. The chelating agent, EDTA, inhibits proteolytic activity on all substrates tested with exception of BAPNA. N-bromosuccinimide (NBS) inhibits only proteolytic activity on BAPNA and congocoll. Chemical analysis for tryptophan and the absorption spectrum (220-340 mμ) of NBS-treated venom indicate a loss of ca. half the tryptophan content (2.44%) of Crotalus atrox venom.

Results of Table 4 (page 13) show the effect of dihyrothioctic acid (DHTA) on caseinolytic activity of Crotalus atrox venom. Low concentrations of DHTA have little effect on caseinolytic activity of high concentrations of venom (72 mg/ml). When higher concentrations of DHTA are mixed with less venom (4 mg/ml), an immediate inhibition of caseinolysis occurs.

TABLE 3

Effect of Various Enzymic Functional Group Inhibitors on Proteolytic Activity of *Crotalus atrox* Venom

Venom and the inhibitors listed were incubated at 37° for 0 and 60 minutes in appropriate buffers then assayed. The final concentrations of venom in the incubation mixture for assay with azocoll, BAPNA, congocoll and casein were 0.5, 1.0, 0.25, and 0.5 mg per ml, respectively (see Materials and Methods).

	Inhibitor Conc.	BAPNA Per Cent Activity		Azocoll Per Cent Activity		Congocoll Per Cent Activity		Casein Per Cent Activity	
	mM								
Inhibitor		0 Min	60	0 Min	60	0 Min	60	0 Min	60
OMIU*	-	121	100	-	83	85	54	62	47
pHMB	0.1	99	79	105	143	100	94	8	9
"	1.0	97	-	125	135	93	96	8	12
IAA	10.0	96	100	99	65	121	92	100	72
"	100.0	97	100	100	65	119	121	0	0
NEM	10.0	128	115	87	84	84	71	39	47
"	100.0	139	103	95	69	66	58	-	-
2-MCE	100.0	107	82	79	72	21	3	7	2
"	1000.0	98	47	26	3	4	3	0	2
BAL	5.0	-	-	21	9	6	0	1	1
"	10.0	100	98	15	-	4	3	3	3
EDTA	0.1	90	92	-	-	75	7	67	0
"	1.0	94	100	0	3	13	1	13	0
NBS	1.0	72	28	100	79	104	93	104	91
"	10.0	35	6	67	61	18	0	-	-
DFP	1.0	63	17	92	81	86	75	105	74
"	5.0	33	18	95	88	73	59	103	86
Control	-	100	98	100	83	100	83	100	75

* Venom (11.25 mg/ml) was incubated for 72 hours at 2°C with a 50:1 excess of O-methylisourea (OMIU). The unreacted OMIU was removed by dialysis at 2°C against distilled water. The OMIU-treated venom was then incubated at 37°C and 0 and 60 minute samples were removed for assay.

TABLE 4

Effect of Dihydrothioctic Acid on Caseinolytic Activity of *Crotalus atrox* Venom

Venom and dihydrothioctic acid were incubated at 37° for 0 and 60 minutes in 0.1 M Tris buffer, pH 8.0, so that the final concentration of venom or dihydrothioctic acid was that listed in the table. Aliquots were then removed, diluted with buffer to give a final concentration of 1.0 mg per ml. The proteolytic assay of Kunitz (27) was used to test venom activity.

DHTA	Venom	Per Cent Activity	
mM	mg/ml	0 Min	60 Min
0.0	40	100	47
2.4	72	54	49
12	40	29	6
18	20	-	2
18	4	7	1

The effect of ε-aminocaproic acid (EACA) on caseinolysis is seen in Table 5. Low concentrations of EACA activate while higher concentrations inhibit caseinolysis.

TABLE 5

The Effect of Epsilon-Aminocaproic Acid on the Caseinolytic Activity of *Crotalus atrox* Venom

Venom, containing the concentration of EACA shown, was incubated for 60 minutes at 37° then assayed for caseinolytic activity using the Kunitz (27) test.

EACA	Venom	Activity	Venom	Activity
Molarity	mg/ml	Per Cent	mg/ml	Per Cent
0.00	1.00	100	0.50	100
0.10	1.00	112	-	-
0.20	1.00	123	-	-
0.58	0.96	151	0.48	204
0.93	0.93	157	0.47	213
1.69	0.85	88	0.42	126
3.33	0.65	58	0.33	45

O-methylisourea (OMIU), a compound which converts ε-amino groups of proteins to guanidine groups, inhibits only the proteolytic activity on casein and congocoll. It is thus apparent that primary amino groups are needed only for the activity of the caseinolytic enzyme in Crotalus atrox venom and activity on congocoll.

Hydrolysis of Synthetic Substrates. Table 6 lists relative activities of Crotalus atrox venom on various substrates. Only N-p-toluenesulphonyl-L-arginine methyl ester (TAME), N-benzoyl-L-arginine methyl ester (BAME), and N-benzoyl-L-arginine ethyl ester (BAEE) are hydrolyzed. No activity could be detected on other substrates listed with ten times the amount of venom used on TAME, BAME, or BAEE.

TABLE 6

Hydrolysis of Synthetic Substrates by the Proteases of Crotalus atrox Venom

Hydrolysis of various synthetic substrates was measured titrimetrically (METROHM COMBITITRATOR 3-D) at pH 7.50 by adding 0.20 ml of venom (1.0 mg per ml) reconstituted in distilled water, to 2.5 ml of a 0.01 M solution of each substrate dissolved in distilled water, adjusted to pH 7.50 immediately prior to the addition of venom. The rates of hydrolysis were constant until almost all of the substrate had been hydrolyzed.

Substrate	Relative Activity
N-p-Toluenesulphonyl-L-Arginine Methyl Ester	1.00
N-Benzoyl-L-Arginine Methyl Ester	1.19
N-Benzoyl-L-Arginine Ethyl Ester	1.04
N-Acetyl-L-Tyrosine Ethyl Ester	0.00
L-Lysine Methyl Ester	0.00
L-Lysine Ethyl Ester	0.00
N-Benzoyl-L-Glycine Methyl Ester	0.00
Hippuryl-L-Lysine	0.00
Hippuryl-L-Arginine	0.00
N-Carbobenzoxyglycyl-L-Phenylalanine	0.00
N-Carbobenzoxy-L-Glutamyl-L-Phenylalanine	0.00
L-Prolylglycine	0.00
Glycyl-L-Proline	0.00

Proteolysis of Fibrin Blue. Fibrin blue, a substrate of pepsin, was not hydrolyzed by Crotalus atrox venom at either pH 2.0 or 4.0. At higher pH values the substrate becomes soluble and cannot be used.

DISCUSSION

Overnight dialysis (16-18 hours) in the cold with 0.05 M Tris buffer, pH 8.0, causes some loss (ca. 10%) in protein of Crotalus atrox venom. Loss in these presumably low molecular weight species of protein is apparently not correlated with a loss in caseinolytic activity because the latter activity actually increases (by ca. 31%) after dialysis. Obviously, some low molecular weight substances which inhibit caseinolytic activity are removed during dialysis. Dialysis with Tris buffer containing 1.0 mM EDTA resulted in a 63% loss in caseinolytic activity, concomitantly, about an 18% loss in protein occurred.

It is presumed that loss in caseinolytic activity during dialysis against EDTA was a result of removal of some metal ion from a protease in venom or formation of an inactive EDTA-metal-enzyme complex which is inactive with respect to proteolysis. Only with BAPNA as substrate was it found that EDTA did not cause inhibition of proteolysis. With all other substrates almost complete inhibition occurred rapidly with very low concentrations of EDTA. Ethylenediaminetetraacetate has been shown to have no effect on tryptic or chymotryptic activity. The lack of inhibition of EDTA on hydrolysis of BAPNA (a tryptic substrate) by Crotalus atrox venom is similar, therefore, to lack of effect shown by EDTA on trypsin (31). Henriques et al.(3) have shown that proteolytic activity of Jararacan venom on benzoyl-L-arginine amide, another tryptic substrate, is not inhibited by EDTA.

In addition to proteases of Jararacan venom, proteases of Habu venom and Agkistrodon piscivorus venom and others, are inhibited by EDTA (9, 10, 32-34). Papain (35) and ficin (36) are activated by chelating agents (EDTA, BAL). There is no similarity between these enzymes and proteases of several Crotalid venoms. Deutsch and Diniz (34) have found that chlorides of strontium, cobalt, barium, manganese, cadmium, magnesium, calcium, and zinc and nitrates of copper and cobalt, in a three-fold excess over the concentration of EDTA (3.4 mM), were ineffective in reversing complete inhibition of venom of Agkistrodon piscivorus. Henriques et al.(3) have demonstrated that caseinase and gelatinase activities of Jararacan venom are also inhibited by EDTA or 8-Hydroxquinoline.

Maeno (21) has presented evidence that hemorrhagic principles of Habu venom are inhibited by EDTA (10^{-3} M). Maeno and Mitsuhashi (12) reported that hemolytic activity of Habu venom is significantly decreased by EDTA but not o-phenanthrolin.

In these studies, addition of up to a two-fold excess of calcium or other metal ions, with respect to EDTA, to venom that had been dialyzed overnight in the cold with 0.05 M Tris buffer, pH 8.0, containing 1.0 mM EDTA was ineffective in reversing inhibition caused by EDTA. If a metalloenzyme-inhibitor complex had existed, one should probably expect a mass-action reversal of inhibition of proteolytic activity by EDTA when this excess of calcium ions had been added after EDTA. It appears that the metal ion was probably completely removed from the protease(s) with concomitant production of an over-all protein structure altered such that addition of metal ions in an attempt to restore proteolytic activity, became impossible.

The pH optima using azocoll, BAPNA, and congocoll as proteolytic substrates are similar to those found by Pfleiderer and Sumyk (7) for three chromatographically separated proteases of Crotalus atrox venom. The pH optima reported by these authors are 8.0 - 8.2 for one protease and 8.8 - 9.0 for the other proteases. In this study, the pH optima using azocoll, BAPNA, and congocoll as substrates for proteolysis were shown to be 8.0, 8.5, and 9.0, respectively, using the same buffer (0.05 M Tris) as Pfleiderer and Sumyk (7).

The pH optima for proteases of several venoms (3, 18) are similar to those of plasmin (37), trypsin (38), chymotrypsin (39), and thrombin (40). Venom proteases differ in this respect from pepsin (41) and the cathepsins (42) which have low pH optima. It was also interesting to note that fibrin blue, a substrate of pepsin (28), was not hydrolyzed at pH 2.0 or 4.0 by proteases of Crotalus atrox venom.

Calcium ions were found to both activate, Figure 9, and stabilize, Figure 8, proteolytic activity with casein as substrate. In the presence of 1.0 - 10.0 mM calcium ions, little autodigestion of proteases responsible for caseinolysis occurs. Loss in proteolytic activity on casein after 60 minutes incubation of venom at 37° in 0.05 M Tris buffer, pH 8.0, can be restored (and stabilized) by addition of calcium ions to a final concentration of 22 mM. The caseinolytic enzymes in Crotalus atrox venom are thus similar to both trypsin and chymotrypsin which are activated and stabilized by calcium ions (43, 44). Henriques et al.(3) have found that caseinolytic activity of Jararacussu venom is increased in the presence of calcium ions while

Ohsaka (6), using Habu venom, found that calcium ions (1.0 mM) activated caseinolytic activity and proteolytic activity on azocoll.

The results shown in Table 2 indicate that proteolytic activity of Crotalus atrox venom of BAPNA is inhibited by magnesium ions. Magnesium ions had a slightly inhibitory effect on proteases of Jararacan venom. Proteolytic activity, using casein, azocoll, or congocoll as proteolytic substrates is increased in the presence of magnesium ions. Manganese ions inhibit proteolytic activity of Crotalus atrox venom on all the substrates tested, except azocoll. The results presented in this paper are similar to those of Ohsaka (6) and Henriques et al.(3).

It is obvious from the data in Figure 10 that not all of the inhibitory metal ions affect caseinolytic activity of dialyzed venom in the same manner. Nickel and cadmium ions have an almost completely inhibitory effect at low concentrations. Copper and mercury ions cause inhibition which is progressively increased by higher concentrations of each ion up to approximately 11 mM. Manganese and zinc ions, although inhibitory, are required in much larger concentrations than any of the four cations mentioned above to give the same amount of inhibition. Nickel, lead, mercury, and copper ions inhibit proteolytic activity of Crotalus atrox venom with all substrates tested except lead, which does not inhibit caseinolytic activity. Deutsch and Diniz (34) have shown that copper, manganese, cadmium, cobalt, and zinc ions inhibit proteolysis of urea-denatured hemoglobin in varying degrees using venom of Agkistrodon piscivorus. From the above discussion, it may be said that proteases of venom (e.g., Jararacan, Habu, Crotalus atrox, Agkistrodon piscivorus) are all inhibited by divalent ions of cadmium, copper, zinc, mercury, cobalt, and manganese, while calcium activates most of these proteases. In these respects, proteases of venom are, in general, similar to trypsin (40, 43, 44) and chymotrypsin (43).

Only caseinolytic activity appears to be inhibited to any great degree by compounds which react with sulfhydryl groups. In this respect, protease(s) in venom of Crotalus atrox are similar to papain (35), collagenase (45), ficin (36), the cathepsins (42), and the Ha- and Hb-proteinases of Habu venom (9, 10).

Use of NBS in determining whether indole residues might be part of the "active site" of biologically active proteins has been well documented (23, 40-42). NBS is quite specific for tryptophan residues, so much so that it has been used to estimate the tryptophan content of several proteins. Results of the present investigation indicate that

proteolytic activity of Crotalus atrox venom with BAPNA and congocoll as substrates is inhibited by NBS. Apparently, certain tryptophan residues are needed in enzymic catalysis in the case of venom proteases, as in the case of trypsin (46), chymotrypsin (47), or bacterial α-amylase (23).

Diisopropylphosphofluoridate inhibits only proteolytic activity on BAPNA. Markwardt and Walsmann (50) have shown that gelatinase activity of venom of Vipera russellii is only slightly (ca. 20%) inhibited by 2.5×10^{-3} M DFP. Plasmin (37), trypsin (51, 52), chymotrypsin (51, 52), and thrombin (53, 54) are also inhibited by DFP. Apparently, the proteolytic activity of Crotalus atrox venom on BAPNA is similar to the above-mentioned enzymes. The Hβ-proteinase of Habu venom is not inhibited by DFP (9).

Proteolytic activity with all substrates utilized appears to be inhibited by organic compounds containing free thiol groups. Dihydrothioctic acid, Table 4, has little effect at low concentrations on high concentrations of venom, but it does have an immediate inhibitory effect when high concentrations are used on low concentrations of venom. BAL has been used by Veeraraghavan (55) and Sawai et al.(56) as an antitoxic agent in animals for different snake venoms. Mercaptoethanol and BAL, however, Sawai et al.(56) claim, are more toxic per se than DHTA, although equally effective in neutralizing toxic effects of Habu venom in vitro.

In this paper it has been shown that BAL inhibits proteolytic activity, except that determined with BAPNA as substrate. Another reducing agent, 2-mercaptoethanol, has a slightly inhibitory effect on the breakdown of BAPNA, but almost completely inhibits proteolysis with the other substrates tested.

With respect to substrate specificity, Table 6, venom of Crotalus atrox apparently lacks carboxypeptidase A or B activity as indicated by lack of hydrolysis of N-carbobenzoxyglycyl-L-phenylalanine and hippuryl-L-arginine or hippuryl-L-lysine. Absence of prolidase or prolinase activity is indicated by failure of venom to hydrolyze glycyl-L-proline and L-prolylglycine. No chymotryptic activity was detected using N-acetyl-L-tyrosine ethyl ester as substrate. Pepsin-like activity was lacking as manifested by negative hydrolysis of N-carbobenzoxy-L-glutamyl-L-phenylalanine. Maeno and Mitsuhashi (57) have listed some similar results for Hβ-proteinase of Habu venom. As shown in Table 6, papain-like activity was apparently absent because of lack of hydrolysis of N-carbobenzoxyglycyl-L-phenylalanine

and N-benzoylglycine methyl ester (58). Plasmic (59) and thrombic (60) activities, Table 6, were lacking in the sense that lysine esters were not hydrolyzed. Seifter et al.(61) have reported that their collagenase preparations will not hydrolyze BAEE. The substrate specificity or proteases of Crotalus atrox venom is thus more probably similar to trypsin than other types of proteases.

The studies thus far discussed point out two items for consideration when one deals with proteolytic activities of snake venom.

First, use of more than one substrate in assaying proteases in the presence of enzymic inhibitors might indicate whether more than one type of proteolytic enzyme is present in a given venom. If only one type of protease were present in Crotalus atrox venom, then the same degree of inhibition should have resulted when any inhibitor was used under similar conditions regardless of the substrate used to measure enzymic activity, assuming only one active center for the enzyme. It therefore appears that Crotalus atrox venom contains four proteases.

Second, absence of proteolytic activity on a given substrate in fractions collected either electrophoretically or chromatographically, might not exclude absence of every type of protease in the material or fractions tested. Ohsaka et al.(5) have shown that Habu venom contains at least five electrophoretic components having proteolytic activity on casein. One component, however, had no activity on azocoll.

The effect of EACA on the caseinolytic activity of venom of Crotalus atrox is similar to that observed on plasmin (62, 63) and trypsin (62, 63). High concentrations of EACA are needed, however, to cause inhibition.

The proteases of Crotalus atrox venom are obviously not acid proteinases, because of their high pH optima. Evidence for the presence of metal proteinases, thiol proteinases, and a serine tryptic-type proteinase, sensitive to DFP, in Crotalus atrox venom has been presented.

SUMMARY

1. Caseinolytic activity seems to be appreciably inhibited by sulfhydryl group-specific reagents. All proteases are inhibited by thiol-containing compounds. Diisopropylfluorophosphate inhibits only proteolytic activity with N-benzoyl-DL-arginine-p-nitroanilide as substrate. N-bromosuccinimide inhibits only proteolytic activity on

congocoll and N-benzoyl-DL-arginine-p-nitroanilide. Ethylenediaminetetraacetate inhibits proteolytic activity on all substrates tested except N-benzoyl-DL-arginine-p-nitroanilide. ε-Amino-n-caproic acid enhances caseinolysis at low concentrations and inhibits at higher concentrations.

2. Various synthetic proteolytic and esterolytic substrates have been tested using Crotalus atrox venom; only N-(p-toluenesulphonyl)-L-arginine methyl ester, N-benzoyl-L-arginine methyl and ethyl esters were hydrolyzed.

3. Calcium ions and other divalent alkaline earths have a general activating effect on proteolytic activity of venom. Heavy metal ions have a general inhibitory effect on proteolytic activity.

4. The pH optima using azocoll, N-benzoyl-DL-arginine-p-nitroanilide, and congocoll as substrates for proteases of Crotalus atrox venom were found to be 8.0, 8.5, and 9.0, respectively.

5. Crotalus atrox venom, dialyzed with Tris buffer, loses about 10% of its protein concomitantly with an increase in caseinolytic activity. Dialysis in Tris containing 1.0 mM ethylenediaminetetraacetate results in loss of both caseinolytic activity and protein.

REFERENCES

1. Holtz, P. and H. W. Raudonat. Uber Beziehunger zwischen proteolytischer Activitat und Blutcoagulierender sowi bradykininfriesetzender Wirkung von Schlangengifte. Arch. Exp. Path. Pharmakol. 229: 113, 1956.

2. Hamberg, U. and M. R. Silva. On the release of bradykinin by trypsin and snake venoms. Arch. Int. Pharmacodynamie, 110: 222, 1957.

3. Henriques, O. B., A. A. C. Lavras, M. Fichman, F. R. Mandelbaum, and S. B. Henriques. The proteolytic activity of the venom of Bothrops jararaca. Biochem. J. 68: 597, 1958.

4. Yang, C. C., C. C. Su, and C. J. Chen. Biochemical studies on the Formosan snake venoms. V. The toxicity of hyappoda (Agkistrodon acutus) venom and enzyme activities. J. Biochem. (Tokyo), 46: 1209, 1959.

5. Ohsaka, A., H. Ikezawa, H. Kondo, S. Kondo, and N. Uchida. Haemorrhagic activities of Habu snake venom and their relations to lethal toxicity, proteolytic activities and other pathological activities. Brit. J. Exp. Path. 41: 478, 1960.

6. Ohsaka, A. Proteolytic activities of Habu snake venom and their separation from lethal toxicity. Jap. J. Med. Sci. Biol. 13: 33, 1960.

7. Pfleiderer, G. and G. Sumyk. Investigation of snake venom enzymes. Biochim. Biophys. Acta, 51: 482, 1961.

8. Mandelbaum, F. R. and O. B. Henriques. Purification and properties of Bothrops protease A. Arch. Biochem. Biophys. 104: 369, 1964.

9. Maeno, H., S. Mitsuhashi, Y. Sawai, and T. Okonogi. Studies on Habu snake venom. 2a. Enzymatic studies on the proteinase of Habu snake venom. Jap. J. Microbiol. 3: 131, 1959.

10. Maeno, H., M. Morimura, S. Mitsuhashi, Y. Sawai, and T. Okonogi. Studies on Habu snake venom. 2b. Further purification and enzymatic and biological activities of Hα-proteinase. Jap. J. Microbiol. 3: 277, 1959.

11. Maeno, H., S. Mitsuhashi, and R. Sato. Studies on Habu snake venom. 2c. Studies on Hβ-proteinase of Habu venom. Jap. J. Microbiol. 4: 173, 1960.

12. Maeno, H. and S. Mitsuhashi. Studies on Habu snake venom. Jap. J. Exp. Med. 32: 55, 1962.

13. Ohsaka, A. Zone electrophoresis studies of Habu (Crotalidae) venom. J. Biochem. (Tokyo), 45: 259, 1958.

14. Yang, C. C., C. J. Chen, and C. C. Su. Biochemical studies on the Formosan snake venoms. IV. The toxicity of Formosan cobra venom and enzyme activities. J. Biochem. (Tokyo), 46: 1201, 1959.

15. Gitter, S., G. Levi, S. Kochwa, A. De Vries, J. Rechnic, and J. Casper. Studies on the venom of Echis colorata. Amer. J. Trop. Med. Hyg. 9: 391, 1960.

16. Yang, C. C., K. C. Kao, and W. C. Chiu. Biochemical studies on the snake venoms. VIII. Electrophoretic studies of banded krait (Bungarus multicinctus) venom and the relation of toxicity with enzyme activities. J. Biochem. (Tokyo), 48: 714, 1960.

17. Kochwa, S., C. Perlmutter, S. Gitter, J. Rechnic, and A. De Vries. Studies on Vipera palestinae venom fractionation by ion exchange chromatography. Amer. J. Trop. Med. Hyg. 9: 374, 1960.

18. Maeno, H. and S. Mitsuhashi. Studies on Habu snake venom. J. Biochem. (Tokyo), 50: 434, 1961.

19. Master, R. W. P. and S. S. Rao. Identification of enzymes and toxins in venoms of Indian cobra and Russell's viper after starch gel electrophoresis. J. Biol. Chem. 236: 1986, 1961.

20. Gitter, S., C. Moroz-Perlmutter, J. H. Boss, E. Livni, J. Rechnic, N. Goldblum, and A. De Vries. Studies on the snake venoms of the Near East: Walterinnesia aegyptia and Pseudocerastes fieldii. Amer. J. Trop. Med. Hyg. 11: 861, 1962.

21. Maeno, H. Biochemical analysis of pathological lesions caused by Habu snake venom with special reference to hemorrhage. J. Biochem. (Tokyo), 52: 343, 1962.

22. Sawai, Y., M. Makino, I. Tateno, T. Okonogi, and S. Mitsuhashi. Studies on the improvement of treatment of Habu snake (Trimeresurus flavoviridis) bite. 3. Clinical analysis and medical treatment of Habu snake bite on the Amami Islands. Jap. J. Exp. Med. 32: 117, 1962.

23. Okada, Y., K. Onoue, S. Nakashima, and Y. Yamumura. Enzyme-antienzyme systems. II. N-bromosuccinimide modified bacterial α-amylase. J. Biochem. (Tokyo), 54: 477, 1963.

24. Brown, R. K., L. Levine, and H. Van Vunakis. Role of N-terminal lysine in antigenic structure of ribonuclease. Fed. Proc. 16: 159, 1957.

25. Dunn. M. S. Casein in Biochemical Preparations, Vol. 1, H. E. Carter (ed.), John Wiley and Sons, Inc., New York, 1949, p. 22.

26. Gornall, A. G., C. J. Bardawill, and M. M. David. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. 177: 751, 1949.

27. Kunitz, M. Crystalline soybean inhibitor. III. General properties. J. Gen. Physiol. 30: 291, 1947.

28. Nelson, W. L., E. L. Ciaccio, and G. P. Hess. A rapid method for quantitative assay of proteolytic enzymes. Anal. Biochem. 2: 39, 1961.

29. Ridwell, E. and W. E. Van Heyningen. The biochemistry of the gas gangrene toxins. 5. The kappa-toxin ((collagenase)) of Clostridium welchii. Biochem. J. 42: 140, 1948.

30. Haverback, B. J., E. Dyce, H. Bundy, and H. A. Edmondson. Trypsin, trypsinogen and trypsin inhibitor in human pancreatic juice. Amer. J. Med. 29: 424, 1960.

31. Green, N. M. and H. Neurath. The effects of divalent cations on trypsin. J. Biol. Chem. 204: 379, 1953.

32. Philpot, V. B., Jr. and H. F. Deutsch. Inhibition and activation of venom proteases. Biochim. Biophys. Acta, 21: 524, 1956.

33. Philpot, V. B., Jr. Activation of venom proteases and reversal of chelating effects by sodium bicarbonate. Proc. Soc. Exp. Biol. Med. 101: 78, 1959.

34. Deutsch, H. F. and C. R. Diniz. Some proteolytic activities of snake venoms. J. Biol. Chem. 216: 17, 1955.

35. Finkle, B. J. and E. L. Smith. Crystalline papain: number and reactivity of thiol groups; chromatographic behavior. J. Biol. Chem. 230: 669, 1958.

36. Bernhard, S. A. and H. Gutfreund. Ficin-catalyzed reactions: the affinity of ficin for some arginine derivatives. Biochem. J. 63: 61, 1956.

37. Mounter, L. A. and B. A. Shipley. The inhibition of plasmin by toxic phosphorus compounds. J. Biol. Chem. 231: 855, 1958.

38. Schwert, G. W., H. Neurath, S. Kaufman, and J. E. Snoke. The specific esterase activity of trypsin. J. Biol. Chem. 172: 221, 1948.

39. Gutfreund, H. and J. M. Sturtevant. The mechanism of the reaction of chymotrypsin with p-nitrophenyl acetate. Biochem. J. 63: 656, 1956.

40. Ronwin, E. Thrombin properties. Canad. J. Biochem. Physiol. 35: 743, 1957.

41. Baker, L. E. New synthetic substrates for pepsin. J. Biol. Chem. 193: 809, 1951.

42. Fruton, J. S. in The Enzymes, P. D. Boyer, H. Lardy, and K. Myrback (eds.)) Academic Press, New York, 1960, p. 233.

43. Green, M. M., J. A. Gladner, L. W. Cunningham, Jr., and H. Neurath. The effect of divalent cations on the enzymatic activities of trypsin and of α-chymotrypsin. J. Amer. Chem. Soc. 74: 2122, 1952.

44. Ronwin, E. Enzymatic properties of bovine plasmin preparations; evidence of similarity to but non-identity with trypsin. Canad. J. Biochem. Physiol. 34: 1169, 1956.

45. Mandl, L., H. Zipper, and L. T. Ferguson. Clostridium histolyticum collagenase: its purification and properties. Arch. Biochem. Biophys. 74: 465, 1958.

46. Viswanatha, T., W. B. Lawson, and B. Witkop. The action of N-bromosuccinimide on trypsinogen and its derivatives. Biochim. Biophys. Acta, 40: 216, 1960.

47. Viswanatha, T. and W. B. Lawson. The action of N-bromosuccinimide on chymotrypsin. Arch. Biochem. Biophys. 93: 128, 1961.

48. Ramachandran, L. H. The use of N-bromosuccinimide in the study of the composition and structure of proteins. J. Sci. Industr. Res. 21: 111, 1962.

49. Rao, G. J. S. and H. R. Cama. Action of N-bromosuccinimide on some mammalian hemoglobins. Indian J. Chem. 1: 443, 1963.

50. Markwardt, F., and P. Walsmann. Versuche zur Klärung des Wirkungsmechanismus der thrombokinaseähnlich wirkenden Komponente des Russell-Vipern Giftes. Thromb. Diath. Haemorrh. 12: 86, 1962.

51. Jansen, E. F., M. D. F. Nutting, R. Jang, and A. K. Balls. Inhibition of the proteinase and esterase activities of trypsin and chymotrypsin by diisopropylfluorophosphate: crystallization of inhibited chymotrypsin. J. Biol. Chem. 179: 189, 1949.

52. Jansen, E. F. and A. K. Balls. The inhibition of β- and γ-chymotrypsin and trypsin by diisopropylfluorophosphate. J. Biol. Chem. 194: 721, 1952.

53. Shen, Y. S. Influence of diisopropylfluorophosphate on the thrombin-like action of snake venoms. J. Formosa Med. Ass. 61: 245, 1962.

54. Miller, K. D. and H. Van Wunakis. The effect of diisopropyl-fluorophosphate on the proteinase and esterase activities of thrombin and on prothrombin and its activators. J. Biol. Chem. 223: 227, 1956.

55. Veeraraghavan, N. Action of some sulfhydryl compounds on cobra venom. Indian J. Med. Res. 39: 267, 1951.

56. Sawai, Y., M. Makino, and Y. Kawamura. Studies on the anti-toxic action of dihydrolipoic acid (dihydrothioctic acid) and tetra-cycline against Habu snake (Trimeresurus flavoviridis HALLOWELL) venom, in: Venoms and Poisonous Animals and Noxious Plants of the Pacific Area, H. L. Keegan and W. V. MacFarlane (eds.)), Pergamon Press, New York, 1963, p. 327.

57. Maeno, H. and S. Mitsuhashi. Studies of Hβ-proteinase of Habu snake venom, with special reference to substrate specificity and inhibitory effect of serum. J. Biochem. (Tokyo), 50: 330, 1961.

58. McDonald, C. E. and A. K. Balls. On the esterolytic activities of papain and trypsin. J. Biol. Chem. 229: 69, 1957.

59. Troll, W., S. Sherry, and J. Wachman. The action of plasmin on synthetic substrates. J. Biol. Chem. 208: 85, 1954.

60. Sherry, S. and W. Troll. The action of thrombin on synthetic substrates. J. Biol. Chem. 208: 95, 1954.

61. Seifter, S., P. M. Gallop, L. R. Klein, and E. Meilman. Studies on collagen. II. Properties of purified collagenase and its inhibition. J. Biol. Chem. 234: 285, 1959.

62. Ablondi, F. B., J. J. Hagan, M. Philips, and E. C. De Renzo. Inhibitions of plasmin, trypsin, and the streptokinase-activated fibrinolytic system by ϵ-aminocaproic acid. Arch. Biochem. Biophys. 82: 153, 1959.

63. Alkjaersig, N., A. P. Fletcher, and S. Sherry. ϵ-aminocaproic acid: an inhibitor of plasminogen activation. J. Biol. Chem. 234: 832, 1959.

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Crotalus atrox venom proteases are inhibited by several functional group-specific inhibitors. Heavy metal ions inhibit, whereas calcium and other divalent alkaline earths have a general activating effect on these proteases. The type and number of proteases in crude venom can be discerned by combined use of these reagents and four different substrates. Two of these substrates, N-benzoyl-DL-arginine-p-nitroanilide and congocoll have recently been introduced as substrates for trypsin, but have not as yet been used to assay proteolytic enzyme activity of snake venoms.

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